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ABSTRACT

The majority of cystic fibrosis patients produce a mutant form of CFTR (ΔF508) which has been shown to be mislocalised in both humans and mice. G480C, another clinically "severe" mutation, has also been demonstrated to be defective in its intracellular processing, but when allowed to traffic in Xenopus oocytes showed similar channel characteristics to that of wildtype CFTR. We have replicated the G480C mutation in the murine *Cftr* gene using the "hit and run" double recombination procedure. As expected, the G480C cystic fibrosis mouse model expresses the G480C mutant transcript at a level comparable to that of wild type *Cftr*. The homozygous mutant mice were fertile, had normal survival, weight, tooth colour and no evidence of caecal blockage, despite mild goblet cell hypertrophy in the intestine. Analysis of the mutant protein revealed that the majority of G480C CFTR was abnormally processed and no G480C CFTR-specific immunostaining in the apical membranes of intestinal cells was detected. The bioelectric phenotype of these mice revealed organ specific electrophysiological effects. In contrast to ΔF508 "hit and run" homozygotes, the classic defect of forskolin-induced chloride ion transport is not replicated in the caecum, but the response to low chloride in the nose is clearly defective in the G480C mutant animals. The mild phenotype of these G480C mutant animals combined with the defective chloride transport in the nose uniquely provides a valuable resource to test novel pharmacological agents aimed at improving trafficking and correcting the electrophysiological defect in the respiratory tract.

INTRODUCTION

Cystic fibrosis (CF) is a fatal autosomal recessive genetic disease characterised by abnormal epithelial ion transport. The mutated gene responsible is the *cystic fibrosis transmembrane conductance regulator (CFTR)* gene encoding a protein which functions among other things as an epithelial cAMP-regulated Cl^- ion channel [1]. Many hundreds of mutations have been described in CF patients but the most common mutation, is caused by deletion of a phenylalanine at position 508 (ΔF508)[2]. This mutation causes misfolding of the protein, incomplete glycosylation and prevents correct trafficking of the protein to the apical plasma membrane[3].

In humans ΔF508 accounts for approximately 70% of all mutant CF alleles and is considered "severe" with respect to cystic fibrosis phenotype. Other "severe" mutations have been described and G480C is one where an amino acid substitution occurs in exon 10 of CFTR[3]. Both G480C and ΔF508 mutations show a primary defect in protein processing and trafficking, such that mutant protein is retained and degraded in the endoplasmic reticulum, resulting in a severe reduction at the plasma membrane[4;5;6]. When expressed in Xenopus oocytes (where the transport block can be overcome), the G480C protein has an apical plasma membrane Cl^- channel activity identical to that of wild type CFTR[6]. Contradictory reports exist for ΔF508 function at the apical membrane, some suggesting reduced function and others suggesting normal function [7-9].

We created mutant mice that carry the G480C mutation by gene targeting using the “hit and run” technique [10;11]. These mice are comparable to the

Cftr^{tm1Eur} mice where the "hit and run" technique was used to introduce the ΔF508 mutation into the mouse. [12]. In both these *Cftr* mutant mice the only alteration in the *Cftr* gene is the introduced mutation, and transcription of the mutant allele does not deviate from wild type levels[13]. This is in contrast to replacement mouse models, where the gene targeting event introduces plasmid sequences into an intron and the transcription of the mutant allele is reduced[14,15]. Thus for example, in the *Cftr*^{tm2Cam} ΔF508 mice the level of Cftr mRNA is approximately 15% of wildtype levels[14]. In such mutant mice (eg. the ΔF508 *Cftr*^{tm2Cam} and the G551D *Cftr*^{tm1G551D} mice), it is therefore difficult to assess whether the phenotype is due to the precise mutation or principally related to a reduction in the level of the transcript.

The *Cftr*^{tm1Eur} mice, where ΔF508 *Cftr* transcript levels are normal, possess a small, residual forskolin response in the gut [12], not evident in the intestine of the "null" or other ΔF508 mutant mice. In the nasal tissue these ΔF508 mutant mice cannot be distinguished from littermates on the basis of their low chloride response [12]. The “null” and low level transcript ΔF508 mice die perinatally or at weaning due to intestinal blockage but do display a clear defect in their nasal bioelectrics. In this study an accurate mouse model of the G480C mutation was used to assess the phenotype of another “severe” cystic fibrosis mutation *in vivo* and to clarify the organ-specific consequences of a mistrafficking mutant.

RESULTS

Generation of *Cftr*^{tm2Hgu} mice which carry the G480C mutation

ES cells modified at the *Cftr* locus to possess the G480C mutation in exon 10 by 'Hit and Run' gene targeting have been previously described [11]. 4 ES cell clones modified to possess the G480C mutation which had normal karyotypes were used for blastocyststst injections. Germline transmission of the mutation was detected by genotyping agouti offspring from mating the chimaeric males to C57Bl/6 females and was observed in 2 independent clones, with all mice used in this study originating from a single transmitting chimaeric founder animal. Homozygous G480C mutant mice are designated *Cftr*^{tm2Hgu} following the Mouse Nomenclature Committee guidelines. A novel *NsiI* restriction enzyme site was created at the site of the G480C mutation (Figure 1A) and restriction enzyme digestion verified the faithful replacement of the wild type exon 10 with the mutant exon 10 (Figure 1 B, C, D). PCR amplification of exon 10 and digestion with *NsiI* differentiated the enzyme resistant wildtype band from the G480C containing band, which can be digested with the enzyme (Figure1E).

G480C mutant mice express the mutant allele at wild-type levels

Both male and female mice heterozygous for the G480C allele were included in the study and a range of tissues investigated. The ΔF508 mice generated by replacement gene targeting (*Cftr*^{tm2Cam}) show differences in the extent of allele bias between tissues[14]. *Cftr* mRNA expression was demonstrated by RT-PCR between exons 9 and 10 and was observed in all of the tissues investigated, (lung, colon, jejunum, ileum and testis). Samples were taken during

the linear range of the PCR amplification as determined by real-time luminescence assay (Methods), to reduce heteroduplex formation. G480C products were distinguished from wild type by the presence of the novel *NsiI* restriction site polymorphism engineered adjacent to the missense change. Densitometric quantitation of PCR products after *NsiI* digestion indicated approximately equal ratios of each allele in the ileum, jejunum, testis and lungs (Figure 2 and Table 1), except in one lung sample which appeared to undergone degradation following digestion. These results indicate that the modified allele was expressed at the same level as the wild type allele and therefore the presence of the G480C mutation has no effect on the level of expression.

G480C CFTR is incompletely processed

Analysis of CFTR processing in isolated jejunal enterocytes of wild-type mice by Western blotting demonstrated a normal pattern of CFTR isoforms with the core-glycosylated isoform of CFTR (band B) in the ER, and the mature, fully-glycosylated isoform (band C) in the plasma membrane. Band B was identified only in the crude membrane preparation (Fig. 3, lane 1) consisting of a mixture of ER, Golgi and plasma membranes, whereas band C was additionally detected (and approximately two-fold enriched) in the brush border membrane vesicle preparations (BBMV) (Fig.3, lane 3) consisting of virtually pure apical membranes. In contrast, intestinal epithelium from homozygous G480C mice showed a normal intensity of the B-band of CFTR in the crude jejunal membrane fraction (Fig.3, lane 2), but a strongly reduced intensity of the C-band (measured as 8% (+/-2) n=4, residual CFTR compared to wildtype as determined by dilution

calibrated scanning of the bioluminescence) in the BBMV (Fig.3, lanes 2 and 4). This outcome clearly demonstrates that the G480C *CFTR* mutant protein is retained in the ER of the enterocytes *in vivo* and that only a very small fraction is able to escape the quality control mechanism in the ER and reach the cell surface.

Immunocytochemical detection of CFTR in the jejunum from wild-type mice using the R3195 antibody, (in keeping with previous findings [13], showed intensive staining of the apical region of all crypts (Fig. 4A). In addition, due to the improved sensitivity of CFTR staining in microwave-treated paraffin sections as compared to cryosections, CFTR immunoreactivity was also prominent in the brush border membrane of lower- and mid-villus cells. In contrast, CFTR expression in crypts and villi from homozygous G480C mutant mice remained below the detection level of the immunocytochemical technique (Fig. 4B). This finding confirms the results of the Western blotting shown in Fig. 3 and is in line with the concept of a processing defect affecting the maturation of G480C-*CFTR* in both the crypt and villus compartments.

Phenotype of the cystic fibrosis mutant mice homozygous for the G480C mutation

Figure 5 demonstrates that genotypes of the litters produced from matings between G480C heterozygous mice did not deviate from the expected Mendelian ratio of wild type:heterozygotes: homozygotes of 1:2:1 and no reduction in the number of homozygotes was observed. This was true in the offspring from mating heterozygotes from the outbred 129/Ola/C57Bl/6N background and also

from mating heterozygotes from a 4th generation of backcross matings to C57Bl/6N mice. Further, homozygous G480C mice did not show any increased mortality over wild type animals (pre or post weaning) over an 18 month period. The weight (at weaning) of the three genotypes did not differ significantly (figure 5), and both males and females were fertile (data not shown).

Histological analysis of intestinal sections demonstrated focal hypertrophy of goblet cells in the G480C homozygous mutant mice. Sections from the G480C mutants could easily be distinguished from wild type by this criterium alone (figure 4). This hypertrophy is however much less severe than that observed in the "null" (*Cftr*^{tm1Unc} or *Cftr*^{tm1Cam} [16;17]) mice and is similar to that observed in the *Cftr*^{tm1Eur} ΔF508 mice[12]. In addition we saw no evidence (n=20 mice) of dramatic distention of crypts in the small intestine, blockage, nor any evidence of an abnormal coiled, worm-like caecum impacted with sticky fecal material which is described as the only obvious intestinal abnormality in the *Cftr*^{tm1Unc} mice sacrificed while apparently still healthy[16].

The *Cftr*^{tm1Unc} "null" mice and *Cftr*^{tm1Eur} ΔF508 "hit and run" mice display white teeth as a result of decreased enamel mineral content and increased magnesium ([18] and H de J unpublished data). Both these strains of mice can be genotyped purely on the basis of their white tooth colour. The incisor teeth of the G480C mutant mice however were not abnormally white (data not shown).

Electrophysiological characteristics of G480C *CFTR* mice

The reduced chloride permeability of the epithelium due to CFTR dysfunction, causes typical abnormalities in the ion transport of different epithelia in both CF

individuals and *Cftr* mutant mice. The G480C cystic fibrosis mice do not suffer from the intestinal blockage (the first signs of which are located in the caecum) that is seen in mice with a complete disruption of *Cftr* expression, so we examined the electrophysological profile of these animals in the intestine.

In the caecum, Ussing chamber measurements revealed that the initial baseline Isc (short circuit current) was significantly ($p=0.0001$) reduced in the G480C homozygous mutants compared to controls (Fig 6A). However the response to forskolin, which activates CFTR through an increase in cAMP, was not significantly different between wild type and mutant animals. Carbachol (which stimulates Ca^{2+} mediated chloride transport) probably also via CFTR in the intestine, was however significantly ($p=0.01$) reduced compared to wildtypes. In the jejunum (Figure 6B) however, the baseline Isc was not significantly different between the mutants and wildtypes, but the forskolin response was significantly ($p=<0.01$) different. The carbachol response, as in the caecum, was significantly reduced compared to littermates ($p<0.01$).

Several studies have demonstrated that in the intestine of rodents, the baseline and stimulated secretions are independent when the stimulation is made with forskolin. Naturally cAMP levels may be elevated via prostaglandin increase. These studies however show that the forskolin- and prostaglandin-mediated pathways are independent in as far as normal forskolin-mediated secretion can be achieved following prostaglandin-mediated secretion [19 20;21]. In addition we can demonstrate (H.de J. unpublished results) that the basal short circuit current in the murine intestine was not affected by 10^{-5}M indomethacin (a

potent inhibitor of prostaglandin synthesis) and is therefore not prostaglandin/cAMP mediated. We are therefore confident that the responses generated in this study by forskolin are independent of any prostaglandin-mediated baseline secretion in the jejunum and caecum.

We also examined the nasal biolelectrics of these mice (figure 6C) and in common with all other described CFTR mutant mice[22], the baseline was significantly (p=<0.001) raised. The response to a low chloride gradient was significantly (p=0.0001) reduced in the G480C mutant animals, in contrast to the normal response reported in the *Cftr*^{tm1Eur} ΔF508 mouse.

DISCUSSION

The mutant mice we present here mimic human CF individuals with the "severe" G480C mutation. The G480C mutant protein was detected in a pancreatic insufficient African-American CF patient [6]. Western blot analysis and immunofluorescent analysis revealed that even when overexpressed in 293T cells, no fully glycosylated nor apically localised protein was detectable. This suggested that the G480C protein was similar to the ΔF508 protein and subject to defective intracellular processing. We demonstrate that when replicated in the mouse, the G480C mutant CFTR is mislocalised and the defect in chloride ion transport characteristic of CF varies between tissues and is present in the nose and jejunum but absent from the caecum with no evidence of fatal gut blockage.

G480C mutant mice express normal levels of the mutant allele.

The "hit and run" procedure used to generate these mice results in the only genomic alteration being at the site of the mutation. It follows that the

message levels of the mutant allele will be equivalent to the wild type allele. In ΔF508 / wild type heterozygous humans, mRNA of both alleles is present at an equal ratio [23]. Mice created by gene targeting for the common ΔF508 mutation by replacement gene targeting[14], used a vector which included a reverse orientation *HPRT* mini-gene selection cassette in intron 10 along with the ΔF508 mutation in exon 10. The derived heterozygote mice were determined to have a *Cftr* expression bias at the mRNA level in favor of the wild type allele over the engineered ΔF508 allele. No reasons for this bias were investigated or considered in the original paper, but the most likely possibility is interference from the transcription of *HPRT* in the reverse orientation, from within intron 10; although binding of transcription repressors, chromatin modification, methylation and RNA processing differences are all possibilities. These *Cftr*^{tm2Cam} ΔF508 mutant mice have a very low level of mutant transcript and a phenotype very similar to the "null" *Cftr* mutant mice where the vast majority die perinatally or around weaning from intestinal blockage. The *Cftr*^{tm1Kth} ΔF508 mice were also made by replacement gene targeting and again have a high level of early death from intestinal blockage[24]. The "hit and run" (*Cftr*^{tm1Eur}) ΔF508 mutant mouse in contrast has a normal level of mutant transcript and although it displays evidence of growth retardation at weaning [12], it does not demonstrate a phenotype of death from gut blockage. In contrast to the ΔF508 mice generated by replacement gene targeting, the *Cftr*^{tm1Eu} ΔF508 'hit and run' mice and the *Cftr*^{tm2Hgu} G480C 'hit and run' mice generated here both express normal levels of the mutant allele.

G480C mutant protein is mislocalised

The majority of G480C CFTR when subjected to Western blot analysis is clearly mislocalised *in vivo* in the mouse intestine. However a low (approximately 8% of wild type) level of mature band C is evident in the BBMV preparations. This outcome clearly suggests that the majority of G480C-CFTR mutant protein is retained in the ER of the enterocytes *in vivo* but that a significant fraction is able to escape the quality control mechanism in the ER and travel to the cell surface. In jejunal BBMV samples from ΔF508 *Cftr*^{tm1Eur} mice the residual level of mature protein was estimated at 3%(+/-1) of wild type, (HdJ, RW unpublished results). This suggests that the G480C processing defect in the intestine is slightly less severe than that of the ΔF508 mutant *in vivo*. It should be noted that although 8% of normal levels of mature G480C was detectable in Western blot analysis of BBMV, only cytoplasmically localized protein could be detected by immunohistochemistry.

The phenotype of the Cftr^{tm2Hgu} G480C mutant mice is mild

The *Cftr*^{tm2Hgu} G480C mutant mice do not demonstrate a phenotype of death from gut blockage and unlike the ΔF508 *Cftr*^{tm1Eur}mice do not even display any evidence of growth retardation at weaning. The histology of the G480C intestine is not severely abnormal unlike the *Cftr*^{tm1Unc} "null" mice, which display extensive goblet cell hyperproliferation, increased mucus accumulation and luminal obstruction. The ΔF508 mice display focal hypertrophy of goblet cells in the crypts of the small intestine. The G480C mice (as is evident in fig 4), also do not have any gross abnormalities but do display a mild focal hypertrophy of

goblet cells comparable to the data reported for the ΔF508 *Cftr*^{tm1Eur} homozygotes.

The classic CF chloride transport defect is not present in the caecum and may account for the lack of intestinal blockage.

The G480C mutant mice do not show a defect in their forskolin response in the caecum, although baseline and carbachol response are altered compared to wild type. The ΔF508 “hit and run” mutant mice in contrast to the G480C mice have a significant but markedly reduced (by 85%) forskolin activated chloride ion conductance in the caecum compared to wild type[13]. In fact all previously reported CF mutant mice that show a reduced incidence of death from intestinal obstruction (*Cftr*^{tm1Hgu},*Cftr*^{tm1Eur}, *Cftr*^{tm1G551D}), also show an increased caecal cAMP response compared to *Cftr* mutant mice with a high death rate from intestinal blockage [22]. The caecum is the region of the gut where most of the intestinal blockage occurs in “null” *Cftr* mice so a normal forskolin response (but not baseline or Ca^{2+} agonist response) in this tissue is likely to be important in ameliorating this phenotype.

Variation between the bioelectric phenotypes of ΔF508 and G480C "hit and run" mutant mice could be explained by the effect of modifier genes of residual chloride secretion present in the genetic backgrounds on which the mutations have been bred. This is unlikely because French et al [13] and ourselves (data not shown) have demonstrated that the electrophysiological profiles in the caecum, ileum and jejunum of wild type 129 and C57Bl/6 mouse strains are not significantly different. In addition neither appear to have any dominant gene

effects on the intestinal bioelectrics. Finally, the G480C mice show 100% survival on a mixed 129/C57Bl/6J background (same as that reported for the *Cftr*^{tm1Eur} mice with 100% survival) and this does not alter after 4 backcrosses onto the C57Bl/6J background. This is the same genetic background as that reported for the *Cftr*^{tm1Unc} "null" mice with only 20% survival, and reproduced in our animal house [15].

It is probable that the normal forskolin response in the G480C mice compared to the abnormal response in *Cftr*^{tm1Eur} ΔF508 mice is due to slightly more G480C (3% versus 8%) being correctly processed and reaching the apical membrane. However the results from Xenopus oocyte experiments using human *CFTR* mRNA, strongly suggested that this observed difference between the G480C and ΔF508 response to forskolin was consistent with a trafficking/processing defect in G480C CFTR, and an additional conductance defect in ΔF508 CFTR[6]. We doubt if this additional conductance defect is the case here as the ΔF508 "hit and run" mice have identical channel characteristics to wildtype in the gall bladder epithelia [13].

The fact that “hit and run” ΔF508 mutant mice (*Cftr*^{tm1Eur}) display runting but no evidence of the fatal intestinal blockage presented by “null” CF mice is in contrast to ΔF508 homozygous patients, who do not have reduced incidence of meconium ileus when compared to patients with “null” mutations. One possible explanation is that the G480C and ΔF508 mouse phenotype appear to be different to "null" mice because of differences in human/mouse physiology and

gut architecture, and the mouse is more sensitive to small increases in CFTR function.

Electrophysiological phenotype of the murine G480C mutant protein varies between tissues.

An unexpected finding was the organ-specific differences in CFTR related electrophysiology in the mutant mice. As discussed above we suggest that a normal cAMP-mediated chloride secretion in the caecum is due to around 8% correctly localized CFTR. This agrees with our previous work [25], where we demonstrate, using intercrossed mutants, that a very low level of functional CFTR (5%) is sufficient to have a disproportionate effect on chloride ion transport and a major effect on phenotype (survival due to lack of intestinal obstruction). Interestingly the caecal baseline and Ca^{2+}-activated chloride response remain abnormal despite a normal forskolin response. This implies that these aspects may not be key markers of intestinal blockage and in addition, that there is a CFTR dose-response in terms of restoration of function within an organ. These are important considerations when monitoring the efficacy of therapies aimed at amelioration of the phenotypes in CF individuals.

In the nose of the *Cftr*^{tm2Hgu} mice, in common with all other reported CF mice and individuals[22], the baseline PD is raised indicating a defect in sodium absorption. The familiar defect in cAMP mediated chloride secretion was also observed in the nose, which is in direct contrast to the *Cftr*^{tm1Eur} ΔF508 mutant mouse but in common with all other mutant mice including the 10% residual function mouse (*Cftr*^{tm1Hgu}). So, the fact that the cAMP stimulated response is not

defective in the caecum, but the nasal low chloride response is defective, again demonstrates inter organ differences in this "dose response". This must reflect either tissue specific alterations in the level of mature G480C CFTR with organ-specific subtle translational/post translational differences, or compensatory pathways altering the bioelectric phenotype. We tentatively suggest that correction may be more difficult in the airways as compared to the large intestine.

The Cftr^{tm2Hgu} G480C mutant mouse is a valuable tool for therapy testing

Both the defects in sodium absorption and in chloride secretion are evident in the nose of the G480C mutant mouse and this is widely held to be the mouse tissue that mimics the human respiratory tract phenotype most closely[26]. 70% of CF chromosomes have the ΔF508 mutation and so the vast majority of CF individuals possess at least one ΔF508 allele. This makes pharmacological strategies aimed at improving the function of the ΔF508 mutant CFTR highly relevant. The three mouse models generated to date that express the ΔF508 mutant allele either suffer a high level of intestinal blockage and death (*Cftr^{tm2Cam}*, *Cftr^{tm1Kth}*), or survive well but do not have the bioelectric defect of chloride ion transport in the respiratory tract. The fact that this G480C mutant mouse combines a mistrafficked CFTR mutation (similar to the ΔF508 CFTR) with normal survival means that it is an excellent *in vivo* model for testing drugs aimed at mutant CFTR relocation strategies.

In conclusion, the introduction of the G480C mutation into the mouse *Cftr* gene, using the "hit and run" technique mimics the human allele with normal levels of *Cftr* mRNA production. This has allowed us to demonstrate that the

majority of the G480C mutant protein is mislocalised, but a low level of mature CFTR is detectable by immunoblot. The phenotype of the mutant animals is extremely mild, and does not include severe gut blockage or growth retardation. The G480C homozygous mutant protein has different ion transport effects in different organs with pronounced effects on the baseline in the nose and the caecum. In the nose, the mutant animals have increased absorption whilst in the caecum reduced secretion is evident. Reduced stimulated chloride secretion has been found in the nose and the jejunum but a normal response was found in the caecum and this is most likely responsible for the lack of fatal intestinal blockage and normal weight of the G480C mutant mice.

MATERIALS AND METHODS

Gene Targeting in ES cells

Targeting methodology is as previously described[11]. Screening of litters for transmission of the G480C allele was performed by PCR using 25 base pair primers used to amplify exon 10 from positions 1530-1720 in the *Cftr* gene previously described[27] followed by *NsiI* digestion.

Real-time RT-PCR analysis

Cftr$^{tm2Hgu/+}$ heterozygous G480C mice were sacrificed by CO_2 asphyxiation. Tissues were dissected out, washed in PBS and snap frozen in liquid nitrogen. Samples were thawed, homogenized in 1ml RNAzol B (Biogenesis) and 100 μl chloroform added for phase extraction. Nucleic acids were ethanol precipitated at 4°C, washed with 80% (v/v) ethanol and resuspended in 200 μl DEPC treated dH_2O for 18 hours at 4°C. Yield was determined spectrophotomerically after heat denaturation of 1/250 sample dilutions. $OD_{260/280}$ ratio of 2 was typically obtained. 1μg total cellular RNA was reverse transcribed in 20 μl total volume reactions containing AMV RTase (Boehringer Mannheim) and manufacturers recommended concentrations of buffer, Mg^{2+}, dNTP, gelatin and RNase inhibitor. Random hexanucleotides were used to prime synthesis. Additional to manufacturers recommendations, a 10 min room temperature incubation was included to allow primer annealing and reverse transcription was carried out at 42°C for 1hr. Enzyme was heat killed by incubation at 95°C for 15 min and samples stored at -70°C. For each reverse

transcription reaction carried out, a reverse trancriptase negative control was also performed.

Measurements of laser activated fluorescence of nucleic acid intercalated SYBR Green (FMC Bioproducts) were used to define a reproducible window in which PCR products were sufficiently abundant to be detected by Southern blot, but still within the exponential range of amplification. Reactions were carried out in Idaho Technologies “Light Cycler”. Thermal cycling proceeded through 95°C for 0 seconds (subsequent to a 30 second initial denaturation), 57°C for 0 seconds and 74°C for 6 seconds; the rapid ramping of temperature achievable through the use of glass capillary tubes as reaction vessels. Cycling was performed until the target window of amplification was attained. At this point the temperature was held at 74°C at the end of the 6 second normal extension to allow removal of samples which were slowly cooled to room temperature. Semi-quantitative RT-PCR and appropriate controls were amplified using primers M9AI (5'-AGCAATGGTGACAGAAAACATTCC -3') and M10B (5’-CTGCTGTAGTTGGCAAGCTTTGAC -3’). Reagents were as recommended (Biogene). AmpliTaq (Perkin Elmer) was the polymerase used. PCR products were electrophoretically separated at 100 V on 3% (w/v) agarose gels after digestion to completion with NsiI. Gels were capillary blotted onto nylon transfer membrane (MSI), baked at 80°C for 1 hour and UV crosslinked with a Stratalinker (Stratagene). Hybridization of the ^{32}P end labeled 24mer oligonucleotide M10BI (5’-TCATCATAGGAAACACCAAAGATG-3’) was performed overnight at 50°C and membranes washed in 4×SSC at 50°C.

Densitometric quantitation of signals was determined by phosphoimager and using the Image Quant software area integration algorithm (Molecular Dynamics).

Western blot analysis

Wild-type mice and littermate mice (backcrossed for 4 generations onto the C57Bl/6 strain background) carrying the G480C mutation were anaesthesized with a hypnorm/diazepam mixture. Their abdomen was opened and their small intestine dissected. Epithelial cells originating principally from the villus region were isolated at 0-4^{0}C from the jejunum by everting the intestinal segments on metal rods attached to a vibration apparatus (Vibromixer type E1 from Chemap A.G., Mämmedorf, Switzerland) and exposing them to vibration (50Hz, amplitude 1.5 mm) for 30 min in 0.14 M NaCl containing 5 mM EDTA (pH 7.4). Detached jejunal enterocytes from 2 mice were collected by centrifugation at 800 g for 15 s and suspended in 10 ml of a medium containing 12 mM Tris-HCl (pH 7.4), 0.3 M mannitol, 10 mM KCl, 0.5 mM EDTA and a protease-inhibitor cocktail containing 0.3 mM Pefablock (Boehringer Mannheim, Germany), 10 μg/ml aprotinine, 5 μg/ml leupeptine, 1 μg/ml pepstatin A, 1 μg/ml chymostatin, 50 μg/ml soybean trypsin inhibitor, and 0.03 g/l phosphoramidon. Vesiculation of intestinal membranes was achieved by a freeze-thaw procedure described initially for rat enterocytes [27] and crude microsomal membranes were isolated from half of the cell lysate by a two-step differential centrifugation procedure (10 min, 4.000g, followed by 60 min, 40.000 g). The other half was used to isolate brush border membrane vesicles (BBMV) by differential precipitation with 10 mM

$MgCl_2$ and differential centrifugation (15 min, 3.000g followed by 30 min, 27.000 g) essentially as described [28]. The membrane pellets were solubilized by vortexing in 30 μl of modified Laemmli sample buffer (Tris-HCl 0.06 M; 2% (wt/vol) SDS, 10% (wt/vol) glycerol, 0.1M dithiothreitol, 0.1% (wt/vol) bromophenol-blue and the protease inhibitor cocktail, pH 6.8) and incubated for 30 min at room temperature. Following centrifugation (2 min, 8.000 g) samples of the supernatant (10μl, adjusted to 20 μg protein) were separated on 6% poly-acrylamide slabgels using a Bio-Rad Miniprotean apparatus (Bio-Rad Laboratories, Herfordshire, UK). Proteins were subsequently electroblotted onto nitrocellulose paper (0.1μm pore size, Schleicher and Schuell, Inc., Keene, NH) in 0.025 M Tris, 0.192 M glycine, 20% (vol/vol) methanol. The blots were incubated O/N at 4^0C with 0.02M Tris-HCl, 0.15M NaCl, 0.1% (wt/vol) Tween20, pH7.5 (TTBS), followed by O/N incubation at 4^0C with a 1:1.000 dilution of affinity purified anti-CFTR antibody R3195 in TTBS. Blots were washed three times in TTBS, incubated with peroxidase-conjugated anti-rabbit IgG (Tago Inc., Burlingame, CA; 1:3.000 in TTBS for 2h), and washed four times with TTBS. Peroxidase activity was detected with bioluminescence reagent (ECL kit; Amersham, Braunschweig, Germany) on X-ray film, and CFTR bands were quantitated with the Molecular Imaging System GS-363 (Bio-Rad).

CFTR antibody

The rabbit polyclonal antibody R3195 (kindly donated by Dr. Christopher R. Marino, University of Tennessee, Memphis) was raised against a thyroglobulin-conjugated 13-amino acid COOH-terminal peptide sequence of

rodent CFTR and was affinity-purified on a peptide-epoxide activated Sepharose column, eluted with 4.9M $MgCl_2$, dialyzed and concentrated. CFTR labeling specificity has been demonstrated previously in Western blot and immunocytochemical assays by the loss of immunostaining in tissue specimens from CFTR-/- mice [13].

Immunocytochemical analysis

Wild-type mice and littermate mice carrying the G480C mutation were sacrificed by cervical dislocation, the intestine was dissected and the jejunum was rinsed with ice-cold saline and fixed in 3% (wt/vol) paraformaldehyde for 16 h, prior to standard paraffin embedding. Sections (5-μm) were deparaffinized, followed by microwave treatment in 0.01M sodium citrate solution according to[29]. Endogenous peroxidase activity was blocked by a 30 min preincubation in 0.1M PBS, 0.6% (vol/vol) H_2O_2 and 0.12% (wt/vol) sodium azide. Subsequently, sections were incubated with antibody R3195 (1:100) at room temperature for 1 h followed by a 45 min incubation with a peroxidase-conjugated secondary antibody. Enzymic detection of antigen-antibody complexes was achieved by incubation in substrate solution containing H_2O_2 and 3,3'-diaminobenzidine tetrahydrochloride (DAB, Serva). Finally the sections were counterstained with hematoxylin. Labeling specificity was verified by incubations without primary antibody or without primary and secondary antibody. In both cases background labeling appeared negligible.

Electrophysiological analysis

G480C homozygous animals were assessed *in vivo* (nose) and *in vitro* (jejunum, caecum) and compared with littermate controls.

Jejunum, caecum. These tissues were mounted in standard gas-lift 0.28 cm^2 Ussing chambers at 37^0C under short circuit conditions. Following a 30 min equilibration period the baseline current and transepithelial potential difference were measured and the conductance calculated. Subsequently, amiloride (10 μM, mucosally) was added followed 5 minutes later by forskolin (10 μM, serosally). Carbachol (1 mM, bilaterally) was administered at the plateau of the forskolin response and the chloride component used to assess muscarinic-dependent chloride secretion. Thus in the presence of amiloride, both cAMP-dependent and calcium-dependent chloride secretion were examined.

Nasal. Baseline potential difference and responses to perfused HEPES Krebs low chloride buffer (pH 7.4) in the presence of amiloride (100 μM) were measured as previously described[30]. Briefly a fluid-filled dual channel catheter was placed 5mm within the nasal cavity. The measuring channel and the reference line were connected via calomel electrodes to a hand-held computer (Psion) that provided a simultaneous visual chart presentation (Logan Research Ltd., Rochester, Kent, UK).

Statistics Values are expressed as mean +/- SEM for convenience. The Mann Whitney U test was used to compare groups and the null hypothesis rejected at $p<0.05$.

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References

1. Bear,C.E., Li,C.H., Kartner,N., Bridges,R.J., Jensen,T.J., Ramjeesingh,M., and Riordan,J.R. (1992) Purification and functional reconstitution of the cystic fibrosis transmembrane conductance regulator (CFTR). Cell, 68,809-818.

2. Kerem,B., Rommens,J.M., Buchanan,J.A., Markiewicz,D., Cox,T.K., Chakravarti,A., Buchwald,M., and Tsui,L.C. (1989) Identification of the cystic fibrosis gene: genetic analysis. Science, 245,1073-1080.

3. Kristidis,P., Bozon,D., Corey,M., Markiewicz,D., Rommens,J., Tsui,L.C., and Durie,P. (1992) Genetic determination of exocrine pancreatic function in cystic fibrosis. Am.J.Hum.Genet., 50,1178-1184.

4. Kartner,N., Augustinas,O., Jensen,T.J., Naismith,A.L., and Riordan,J.R. (1992) Mislocalization of delta F508 CFTR in cystic fibrosis sweat gland. Nat.Genet., 1,321-327.

5. Denning,G.M., Ostedgaard,L.S., Cheng,S.H., Smith,A.E., and Welsh,M.J. (1992) Localization of cystic fibrosis transmembrane conductance regulator in chloride secretory epithelia. J.Clin.Invest, 89,339-349.

6. Smit,L.S., Strong,T.V., Wilkinson,D.J., Macek,M., Jr., Mansoura,M.K., Wood,D.L., Cole,J.L., Cutting,G.R., Cohn,J.A., Dawson,D.C., and Collins,F.S. (1995) Missense mutation (G480C) in the CFTR gene associated with protein mislocalization but normal chloride channel activity. Hum.Mol.Genet., 4,269-273.

7. Li,C., Ramjeesingh,M., Reyes,E., Jensen,T., Chang,X., Rommens,J.M., and Bear,C.E. (1993) The cystic fibrosis mutation (delta F508) does not influence the chloride channel activity of CFTR. Nat.Genet., 3,311-316.

8. Drumm,M.L., Wilkinson,D.J., Smit,L.S., Worrell,R.T., Strong,T.V., Frizzell,R.A., Dawson,D.C., and Collins,F.S. (1991) Chloride conductance expressed by delta F508 and other mutant CFTRs in Xenopus oocytes. Science, 254,1797-1799.

9. Dalemans,W., Barbry,P., Champigny,G., Jallat,S., Dott,K., Dreyer,D., Crystal,R.G., Pavirani,A., Lecocq,J.P., and Lazdunski,M. (1991) Altered chloride ion channel kinetics associated with the delta F508 cystic fibrosis mutation. Nature, 354,526-528.

10. Hasty,P., Ramirez-Solis,R., Krumlauf,R., and Bradley,A. (1991) Introduction of a subtle mutation into the Hox-2.6 locus in embryonic stem cells. Nature, 350,243-246.

11. Dickinson,P., Kimber,W.L., Kilanowski,F.M., Webb,S., Stevenson,B.J., Porteous,D.J., and Dorin,J.R. (2000) Enhancing the efficiency of introducing precise mutations into the mouse genome by hit and run gene targeting. Transgenic Res., 9,55-66.

12. van Doorninck,J.H., French,P.J., Verbeek,E., Peters,R.H., Morreau,H., Bijman,J., and Scholte,B.J. (1995) A mouse model for the cystic fibrosis delta F508 mutation. EMBO J., 14,4403-4411.

13. French,P.J., van Doorninck,J.H., Peters,R.H., Verbeek,E., Ameen,N.A., Marino,C.R., de Jonge,H.R., Bijman,J., and Scholte,B.J. (1996) A delta F508 mutation in mouse cystic fibrosis transmembrane conductance regulator results in a temperature-sensitive processing defect in vivo. J.Clin.Invest, 98,1304-1312.

14. Colledge,W.H., Abella,B.S., Southern,K.W., Ratcliff,R., Jiang,C., Cheng,S.H., MacVinish,L.J., Anderson,J.R., Cuthbert,A.W., and Evans,M.J. (1995) Generation and characterization of a delta F508 cystic fibrosis mouse model. Nat.Genet., 10,445-452.

15. Delaney,S.J., Alton,E.W., Smith,S.N., Lunn,D.P., Farley,R., Lovelock,P.K., Thomson,S.A., Hume,D.A., Lamb,D., Porteous,D.J., Dorin,J.R., and Wainwright,B.J. (1996) Cystic fibrosis mice carrying the missense mutation G551D replicate human genotype-phenotype correlations. EMBO J., 15,955-963.

16. Snouwaert,J.N., Brigman,K.K., Latour,A.M., Malouf,N.N., Boucher,R.C., Smithies,O., and Koller,B.H. (1992) An animal model for cystic fibrosis made by gene targeting. Science, 257,1083-1088.

17. Ratcliff,R., Evans,M.J., Cuthbert,A.W., MacVinish,L.J., Foster,D., Anderson,J.R., and Colledge,W.H. (1993) Production of a severe cystic fibrosis mutation in mice by gene targeting. Nat.Genet., 4,35-41.

18. Wright,J.T., Hall,K.I., and Grubb,B.R. (1996) Enamel mineral composition of normal and cystic fibrosis transgenic mice. Adv.Dent.Res., 10,270-274.

19. Aikawa,N., Karasawa,A., and Ohmori,K. (2000) Effect of zaldaride maleate, an antidiarrheal compound, on 16,16- dimethyl prostaglandin E2-induced intestinal ion secretion in rats. Jpn.J.Pharmacol., 83,269-272.

20. Hardcastle,J., Hardcastle,P.T., Ayton,B., Chapman,J., and MacNeil,S. (1992) Calcium-calmodulin-dependent activation of adenylate cyclase in prostaglandin-induced electrically-monitored intestinal secretion in the rat. J.Pharm.Pharmacol., 44,93-96.

21. Cuthbert,A.W., Huxley,C. (1998) The primary and final effector mechanisms required for kinin-induced epithelial chloride secretion. Am.J.Physiol, 274,G578-G583.

22. Davidson D.J. and Dorin, J. R. The CF mouse: an important tool for studying cystic fibrosis. Exp.Rev.Mol.Med. 12-3-2001. http://www-ermm.cbcu.cam.ac.uk/01002551h.htm.

23. Yoshimura,K., Nakamura,H., Trapnell,B.C., Chu,C.S., Dalemans,W., Pavirani,A., Lecocq,J.P., and Crystal,R.G. (1991) Expression of the cystic fibrosis transmembrane conductance regulator gene in cells of non-epithelial origin. Nucleic Acids Res., 19,5417-5423.

24. Zeiher,B.G., Eichwald,E., Zabner,J., Smith,J.J., Puga,A.P., McCray,P.B., Jr., Capecchi,M.R., Welsh,M.J., and Thomas,K.R. (1995) A mouse model for the delta F508 allele of cystic fibrosis. J.Clin.Invest, 96,2051-2064.

25. Dorin,J.R., Farley,R., Webb,S., Smith,S.N., Farini,E., Delaney,S.J., Wainwright,B.J., Alton,E.W., and Porteous,D.J. (1996) A demonstration using mouse models that successful gene therapy for cystic fibrosis requires only partial gene correction. Gene Ther., 3,797-801.

26. Grubb,B.R., Boucher,R.C. (1999) Pathophysiology of gene-targeted mouse models for cystic fibrosis. Physiol Rev., 79,S193-S214.

27. Dickinson, P., Kimber, W.L., Kilanowski, F.M., Stevenson, B.J., Porteous, D.J., and Dorin, J.R. (1993) High frequency gene targeting using insertional vectors. Hum.Mol.Genet., 2,1299-1302

28. van Dommelen,F.S., Hamer,C.M., and de Jonge,H.R. (1986) Efficient entrapment of large and small compounds during vesiculation of intestinal microvilli. Biochem.J., 236,771-778.

29. Devys,D., Lutz,Y., Rouyer,N., Bellocq,J.P., and Mandel,J.L. (1993) The FMR-1 protein is cytoplasmic, most abundant in neurons and appears normal in carriers of a fragile X premutation. Nat.Genet., 4,335-340.

30. Smith,S.N., Middleton,P.G., Chadwick,S., Jaffe,A., Bush,K.A., Rolleston,S., Farley,R., Delaney,S.J., Wainwright,B., Geddes, D.M., and Alton,E.W.F.W. (1999) The in vivo effects of milrinone on the airways of cystic fibrosis mice and human subjects. Am. J. Respir. Cell Mol. Biol. 20,129-134.

Legends to Figures

Figure 1 Generation of G480C Cftr mutant mice by 'Hit and Run' gene targeting.

(A) Targeting vector design. The figure illustrates the genomic structure around exon 10 in mouse ES cells derived from the 129 strain with the filled in box representing exonic sequence. Restriction sites are (A) Asp718I, (E) EcoRI, (H) HindIII, (Hp) HpaI, (Ns) NsiI, and (Xb) XbaI. The structure of the 'Hit and Run' targeting vector, pHRG480C is also shown. The solid line indicates genomic sequence and dotted line indicates vector sequence, the open box represents the mutant exon and the X indicates the site of recombination. 1.3XH indicates the genomic probe used in the Southern blots in Figure 1C and D.

(B) Structure of *Cftr* locus before and after 'Hit and Run' gene targeting. The structure of wild type and 'Hit and Run' gene targeted *Cftr* loci are shown. Wild type *Cftr* genomic structures are shown for strains 129 in which gene targeting was performed, C57 Bl/6 which was used for subsequent breeding and 129 / G480C 'Hit and Run' gene targeted locus. Arrows indicate the size of fragments observed after Southern blot analysis and hybridised with 1.3XH.

(C) Germline transmission of the *Cftr* G480C allele. C57 Bl/6 / 129 Chimeras produced after injection of the targeted ES cell line were bred with C57 Bl/6 females and litters genotyped by Southern blot analysis of Nsi digests probed with 1.3XH. B indicates C57 Bl/6 offspring, C indicates CGR8 parental ES cells, E indicates G480C targeted ES cells used for chimera injection, H indicates

heterozgous *Cftr*$^{G480C/+}$ offspring, 1/B indicates 129 / C57 Bl/6 offspring, M indicates λHindIII molecular weight marker.

(D) Production of homozygous *Cftr*$^{G480C/G480C}$ mice. Heterozygote *Cftr*$^{G480C/+}$ mice were intercrossed and litters genotyped by Southern blot analysis of *NsiI* digests probed with 1.3XH. G indicates *Cftr*$^{G480C/G480C}$ mice, H indicates heterozygous *Cftr*$^{G480C/+}$ mice, W indicates wild-type *Cftr*$^{+/+}$ mice, M indicates λHindIII molecular weight marker.

(E) PCR genotyping of G480C mice. Heterozygote *Cftr*$^{G480C/+}$ mice were intercrossed and litters genotyped by PCR and subsequent *NsiI* digestion. G indicates *Cftr*$^{G480C/G480C}$ mice, H indicates heterozygous *Cftr*$^{G480C/+}$ mice, W indicates wild-type *Cftr*$^{+/+}$ mice, M indicates ϕX174 HaeIII molecular weight marker.

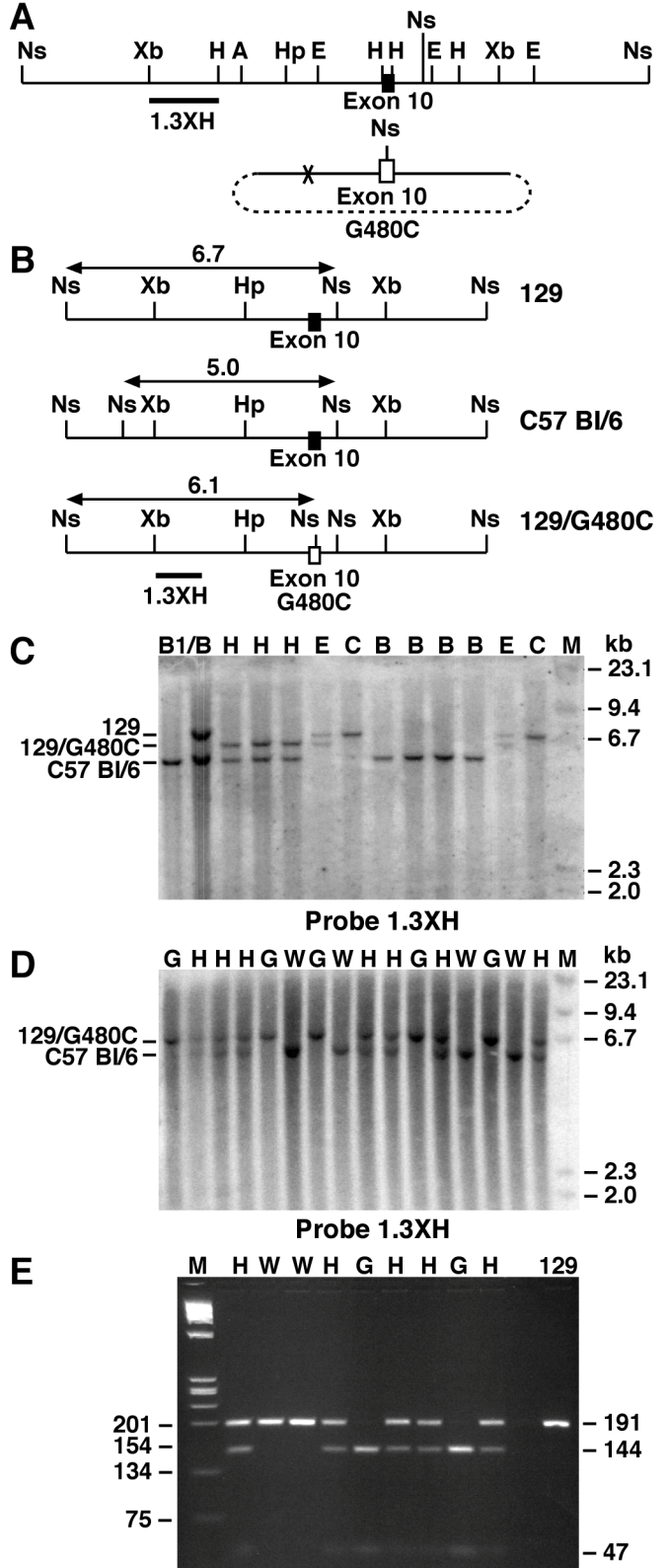
A
Ns
Ns Xb H A Hp E H H E H Xb E Ns
1.3XH
Exon 10
Ns
Exon 10
G480C
B
6.7
Ns Xb Hp Ns Xb Ns
129
Exon 10
5.0
Ns Ns Xb Hp Ns Xb Ns
C57 Bl/6
Exon 10
6.1
Ns Xb Hp Ns Ns Xb Ns
129/G480C
1.3XH
Exon 10
G480C
C
B1/B H H H E C B B B B E C M
kb
23.1
9.4
6.7
2.3
2.0
129
129/G480C
C57 Bl/6
Probe 1.3XH
D
G H H H G W G W H H G H W G W H M
kb
23.1
9.4
6.7
2.3
2.0
129/G480C
C57 Bl/6
Probe 1.3XH
E
M H W W H G H H G H 129
201
154
134
75
191
144
47

Figure 2. Expression analysis of the *Cftr* G480C allele.

A .Real time quantitative RT-PCR expression analysis of the G480C allele was performed. RNA was extracted from tissue samples of heterozygous *Cftr*$^{G480C/+}$ mice and subjected to RT-PCR analysis. RT-PCR products were digested with NsiI and hybridised with oligo M10BI (5'-TCATCATAGGAAACACCAAAGATG-3'). Jej1 and jej2 indicate jejunal sample from 2 independent *Cftr*$^{G480C/+}$ mice, Ile1 indicates ileal sample from mouse 1, +/- R.T indicates +/- reverse transcriptase, C indicates 50:50 wild-type:G480C plasmid PCR control.

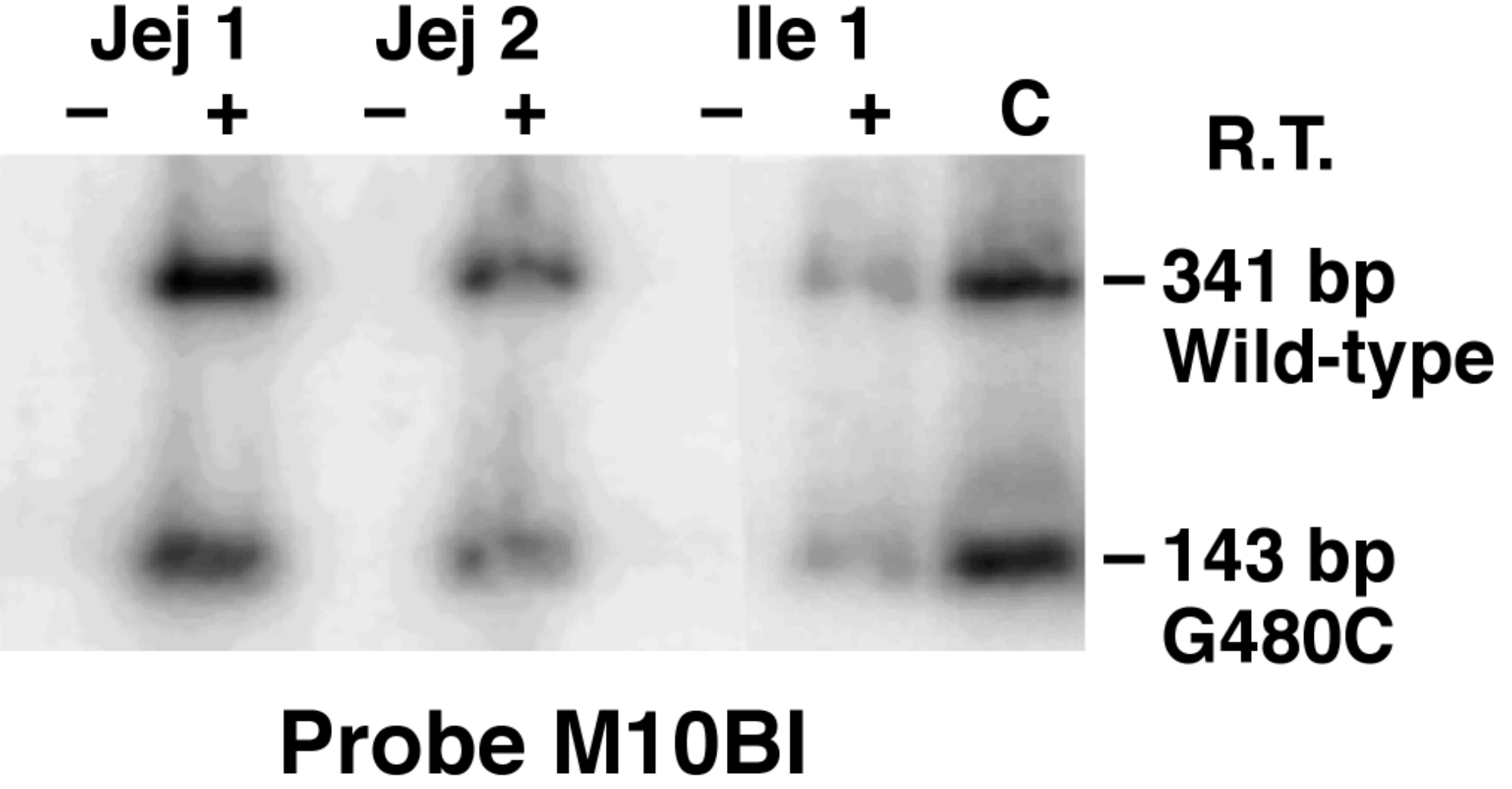
Jej 1
Jej 2
Ile 1
– + – + – + C
R.T.
– 341 bp
Wild-type
– 143 bp
G480C
Probe M10BI

Figure 3 Western blot analysis of CFTR processing in wild-type and G480C *Cftr* mice

Abnormal processing of G480C-CFTR in mouse jejunum. Crude epithelial membranes (lane 1-2) and brush border membrane vesicles (BBMV; lane 3-4) isolated from wild-type (CFTR+/+; lane 1 and 3) or homozygous G480C mutant mice (lane 2 and 4) were subjected to Western blot analysis as described in Methods. Blots were labeled using the CFTR-specific R3195 antibody and the ECL method. Band C refers to mature, complex-glycosylated CFTR, band B indicates the immature, core-glycosylated precursor. Their identity was verified by the finding that both bands were absent in intestinal membranes from CFTR-/- mice (not shown). Data are representative for 3 different experiments involving 5 couples of wild-type and mutant mice.

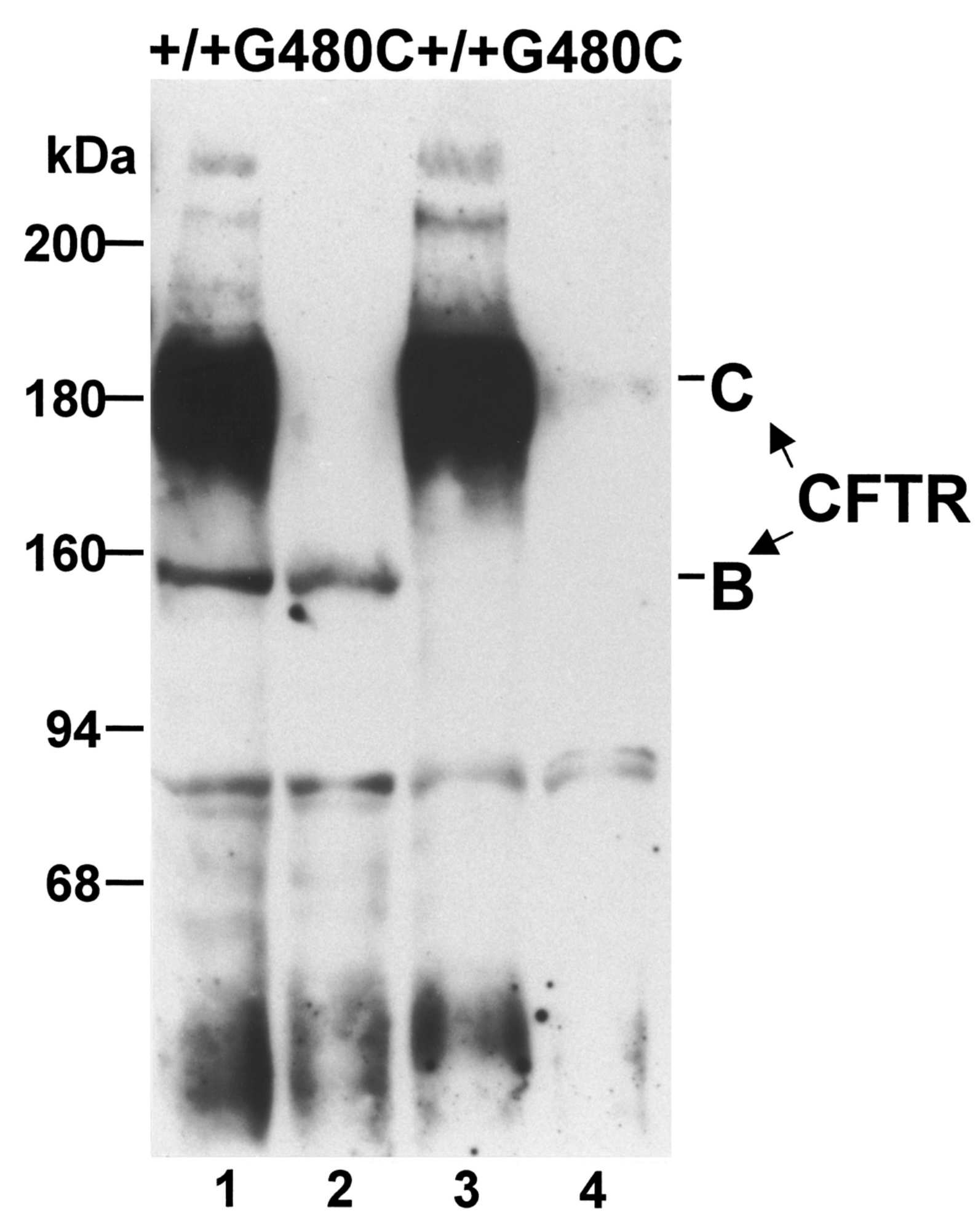
+/+G480C+/+G480C
kDa
200
180
160
94
68
C
CFTR
B
1
2
3
4

Figure 4 Immunohistological analysis of G480C Cftr expression

Immunocytochemical staining of CFTR in the jejunum from wild-type mice (panel A) and homozygous G480C mutant mice (panel B). Immunolabeling with the R3195 anti-CFTR antibody was performed as described in Methods. Crypts and the lower and mid-portion of the villi show intense staining of the apical border of the epithelial cells in wild-type, but not in G480C mouse intestine. Labeling of cells in the lamina propria, presumably representing immune cells (macrophages, lymphocytes), is equally intensive in wild-type and mutant mice (including CFTR-/- mice; not shown) and apparently reflects non-specific, CFTR-independent binding of the primary antibody. Similar differences in CFTR staining pattern were found in 4 couples of wild-type and G480C mutant mice. Examples of goblet cells are indicated by the black arrows.

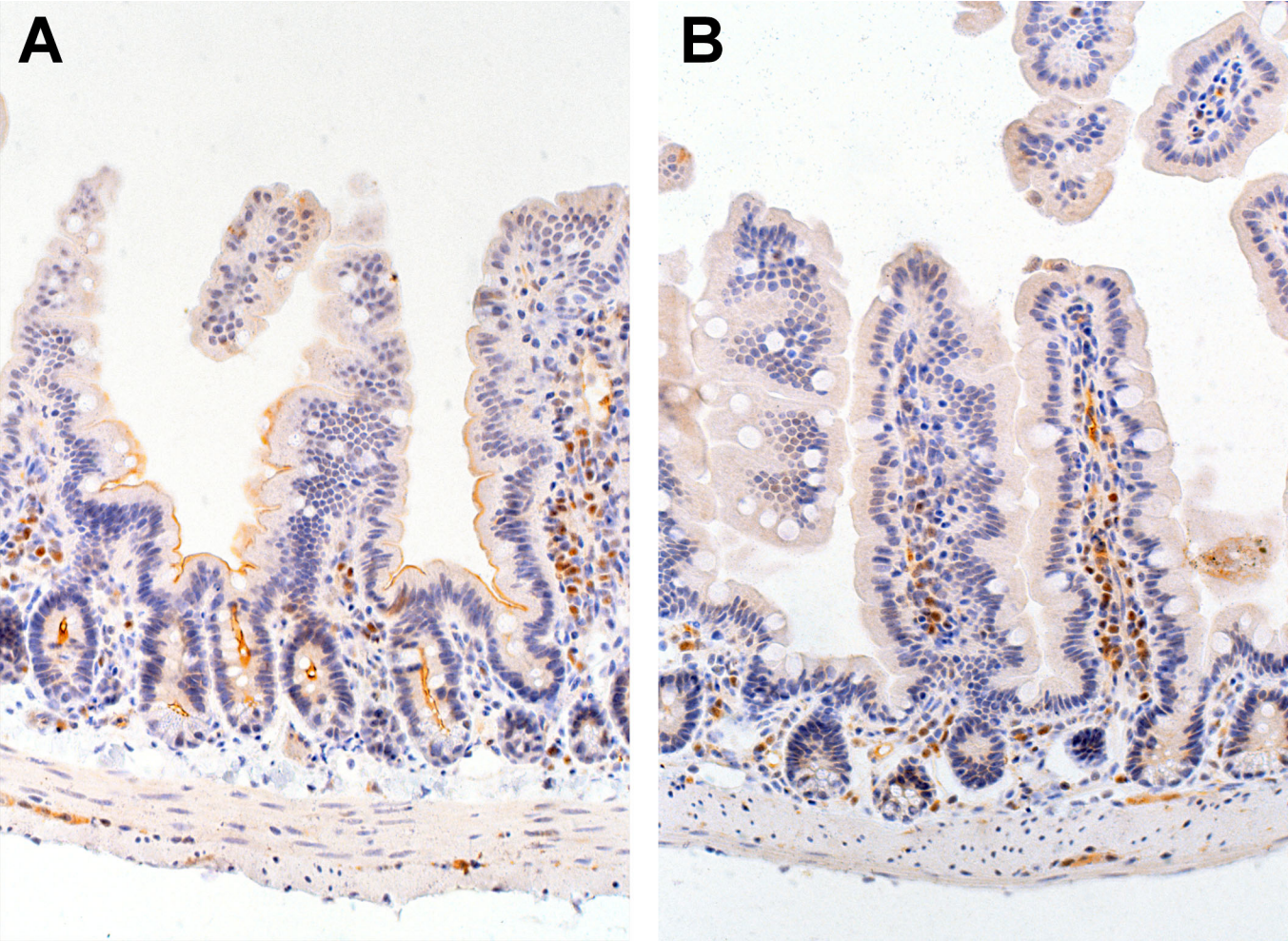
A
B

Figure 5 G480C mice show good survival and no weight reduction

Comparison of weight and number of each genotype at weaning. Mice were generated from *Cftr*^{tm2Hgu}/+ heterozygous crosses. Weight was not statistically different between groups and numbers did not deviate from the expected 1:2:1 Mendelian ratio using Chi square analysis.

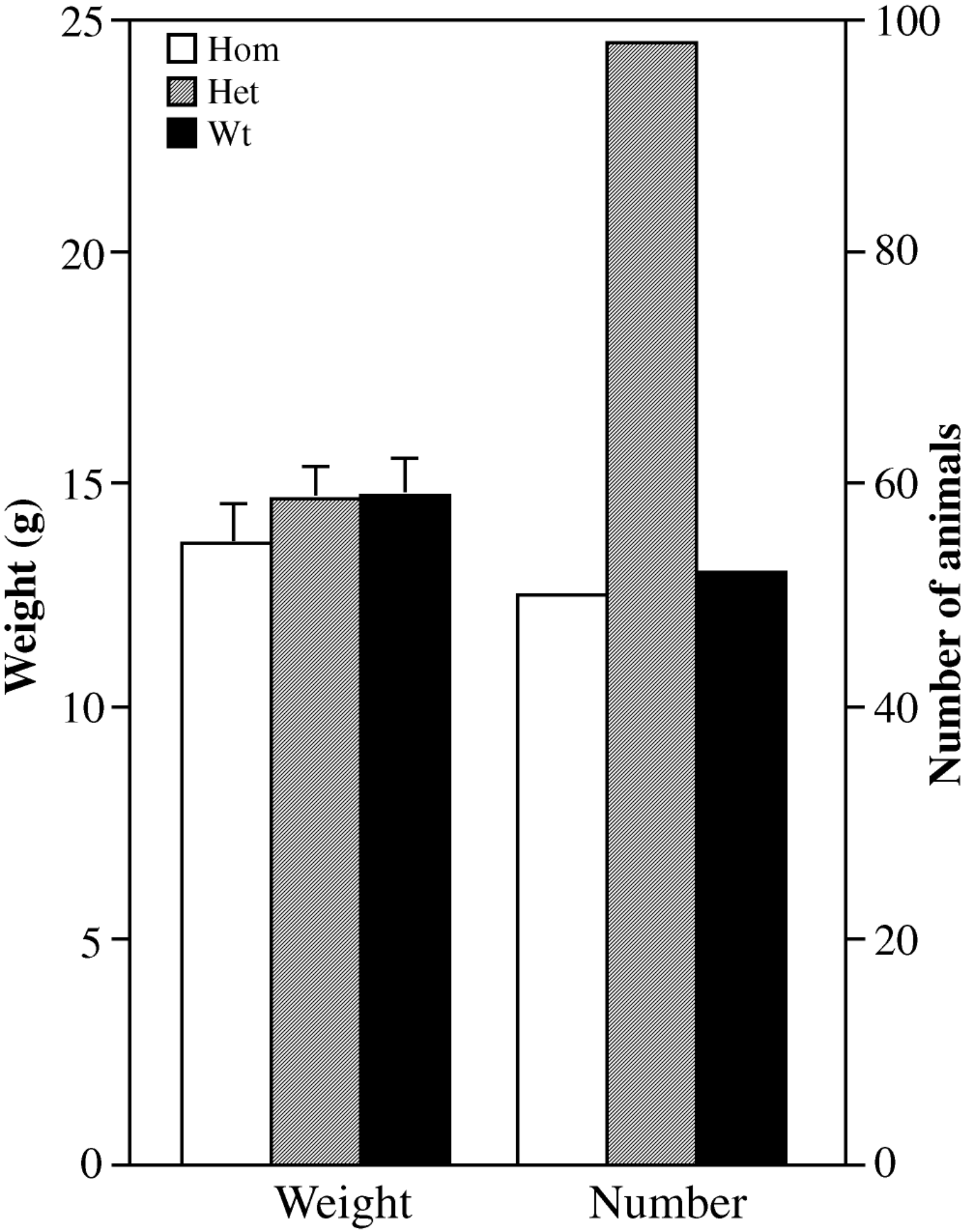
Hom
Het
Wt
25
20
15
10
5
0
100
80
60
40
20
0
Weight (g)
Number of animals
Weight
Number

Figure 6 Tissue-specific bioelectric differences present in the *Cftr*^{tm2Hgu} mice.

Bioelectric characteristics of A) caecum, B) jejunum and C) nose of wildtype (black bars) and G480C *Cftr*^{tm2Hgu} homozygous mutant mice. **** = p=0.0001; *** = p<0.001; ** = p<0.01; * = p<0.05. There was no significant difference in the tissue conductances in either the caecum or jejunum from wild type or mutant animals.

Numbers of animals used, jejunum and caecum baselines and forskolin responses: G480C 12, littermate controls 11, carbachol responses: G480C 6, littermate controls 7; nose baseline: G480C 13, controls 45, nose low chloride: G480C 16, control 38.

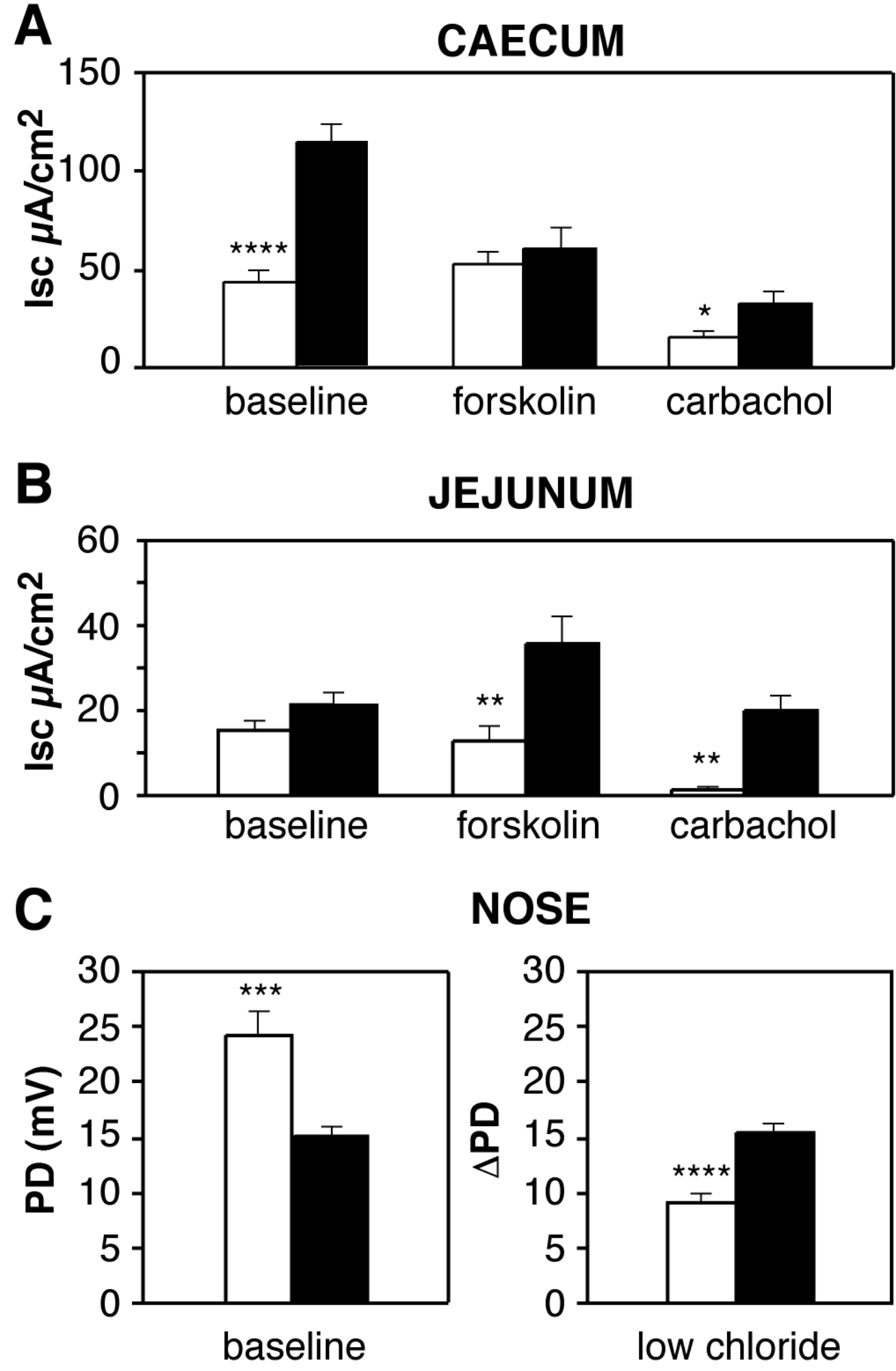
A
CAECUM
Isc μA/cm2
150
100
50
0
****
*
baseline
forskolin
carbachol
B
JEJUNUM
Isc μA/cm2
60
40
20
0
**
**
baseline
forskolin
carbachol
C
NOSE
PD (mV)
30
25
20
15
10
5
0
***
baseline
ΔPD
30
25
20
15
10
5
0
****
low chloride

Table 1

Results from ileum, jejunum and testis show no evidence of an allele bias, only small and inconsistent fluctuations around a 1:1 ratio. Repetitions indicated by the bracketed numbers represent a repetition of the PCR reaction, using the same cDNA stock.

Table 1

Quantification of Expression of G480C allele

Tissue	Mouse	Wild type (%)	G480C (%)
Ileum	1 (rp1)	47.13	52.68
	1 (rp2)	44.55	55.45
Jejunum	1	47.56	52.44
	2	52.13	47.17
Lung	1	20.2	79.8
	2	55.2	44.8
Testis	3 (rp1)	56.77	43.23
	3 (rp2)	56.65	43.35